# APPENDIX 7

tion in the presence of apoptotic stimuli may lower the anti-apoptotic threshold of tumors to provide a more effective treatment against resistant forms of cancer. Additionally, the inhibition of NF-κB function in association with TNF treatment may broaden the limited ability of this cytokine to function in an anti-tumor manner.

*Note added in proof.* Wu *et al.* (*18*) recently demonstrated that NF-κB blocks apoptosis in B cells.

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# Suppression of TNF-α–Induced Apoptosis by NF-κB


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Tumor necrosis factor α (TNF-α) signaling gives rise to a number of events, including activation of transcription factor NF-κB and programmed cell death (apoptosis). Previous studies of TNF-α signaling have suggested that these two events occur independently. The sensitivity and kinetics of TNF-α–induced apoptosis are shown to be enhanced in a number of cell types expressing a dominant-negative IκBα (IκBαM). These findings suggest that a negative feedback mechanism results from TNF-α signaling in which NF-κB activation suppresses the signals for cell death.


The relation between TNF-α signals for NF-κB activation and apoptosis suggests that the two pathways are independent, diverging early in the TNF-α signaling cascade (*1*). Because TNF-α–induced apoptosis is enhanced in the absence of de novo RNA or protein synthesis (*2*), and NF-κB rapidly activates target gene transcription upon TNF-α stimulation, we investigated whether the absence of NF-κB–induced genes alone might enhance TNF-α–induced apoptosis. To test this hypothesis on various cell types, we generated a transdominant-negative mutant of IκBα (*3*). Many signal transduction pathways resulting in NF-κB activation culminate in a serine phosphorylation of IκBα on residues 32 and 36 (*4*). Phosphorylation of the COOH-terminal PEST sequence has been implicated in constitutive turnover of IκBα (*5*). We combined the $NH_2$- and COOH-terminal phosphorylation mutants into a single cDNA (IκBαM) and examined its ability to inhibit NF-κB signaling. We then generated stable transformants expressing IκBαM (*6*) in primary mouse and human fibroblasts, a human lymphoma cell line (Jurkat), and a well-characterized TNF-α–resistant cell line (T24, human bladder carcinoma) (*7*).

Infection with IκBαM retrovirus resulted in a loss of NF-κB inducibility (Figs. 1 and 2). Each cell line represented pools of infected cells to avoid artifacts arising from clonal analysis. Human embryo fibroblasts (HEF) either alone or expressing IκBαM were stimulated for various time periods to examine NF-κB inducibility (Fig. 1). Protein immunoblotting (*8*) (Fig. 1A) shows the expression of murine IκBαM, which migrated faster on SDS–polyacrylamide gel electrophoresis (PAGE) than the endogenous human IκBα (*4*). The expression of IκBαM was only modestly higher than that of endogenous IκBα, demonstrating the abilityof IκBαM to inhibit NF-κB. After TNF-α stimulation in both control and IκBαM cells, endogenous IκBα was phosphorylated and degraded with similar kinetics, demonstrating that in IκBαM-expressing cell lines, the signal transduction pathway upstream of NF-κB activation was not blocked (Fig. 1A). IκBαM was not degraded, presumably because it was not phosphorylated. Because the IκBα gene is induced

**Table 1.** Annexin V–FITC flow cytometric analysis of Jurkat cells stably transduced with IκBαM. Normal or IκBαM Jurkat cells were treated with TNF-α (100 ng/ml) for the indicated times and stained with FITC-labeled annexin V. The cells were then analyzed by flow cytometry as described (*14*). Five thousand cells were analyzed under each condition.

| Time (hours) | Percent annexin V binding | |
|---|---|---|
| | Control | IκBαM |
| 0 | 10.4 | 12.3 |
| 3 | 13.7 | 26.6 |
| 7 | 24.1 | 39.9 |
| 24 | 28.2 | 62.3 |
| 48 | 30.1 | 86.3 |


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by NF-κB (9), inhibition of NF-κB would result in a lack or delay of IκBα resynthesis. Although endogenous IκBα protein was resynthesized in control cells (Fig. 1A), in IκBαM cells, no new IκBα was synthesized. The lack of IκBα resynthesis was not due to induction of cell death because similar results were obtained with a noncytotoxic cytokine, interleukin 1α (IL-1α) (Fig. 1A). Gel mobility-shift experiments with HIV-κB site as a probe (*10*) (Fig. 1B) showed that in control HEF cells after treatment with TNF-α or IL-1α, both the p50/RelA and $(p50)_2$ dimers could be observed, whereas little or no κB binding activity was observed in cells containing IκBαM. Specificity for DNA binding was tested by the use of excess wild-type or mutant κB probe. The same extracts did not effect binding to AP-1 or Oct-1 probes (Fig. 1, C and D). Virtually no NF-κB gel shift was detectable upon activation in a variety of other cell lines (Fig. 2).

Induction of apoptosis in Jurkat and mouse embryo fibroblast (MEF) cells treated for 18 hours with TNF-α was analyzed by staining morphology (*11*) (Fig. 3). Jurkat cells were normally very resistant to TNF-α–induced apoptosis (Fig. 3A), with only 4% showing loss of nuclear structure. In IκBαM-expressing cells, however, there was a pronounced increase in the sensitivity to TNF-α (Fig. 3B), with apoptosis visualized in 44% of the cells. MEF cells showed a similar increase in sensitivity to TNF-α, from 8% in normal MEF cells (Fig. 3C) to 67% in IκBαM-expressing cells (Fig. 3D). An early event in apoptosis is the migration of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (*12*). Annexin V is a protein that binds specifically to PS (*13*). Table 1 compares the values for

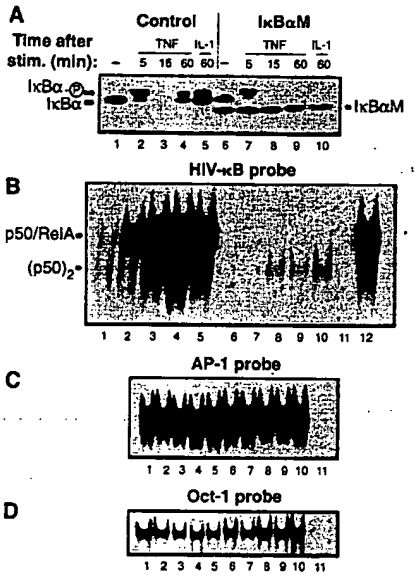


**Fig. 1.** Inhibition of NF-κB in HEF cells by IκBαM. (**A**) Immunoblot analysis of HEF cells stimulated with TNF-α (10 ng/ml) or IL-1α (2 ng/ml). Lanes 1 to 5, normal HEF cells; lanes 6 to 10, cells transduced with IκBαM retrovirus. Cells were stimulated for the indicated times, cytoplasmic extracts were prepared, and 50 μg were analyzed by SDS-PAGE. After transfer to nitrocellulose, the blots were probed with IκBα-specific antiserum. Arrows indicate positions of endogenous IκBα, its phosphorylated form, and IκBαM. (**B**) Gel-shift analysis of nuclear extracts prepared from the same cells as in (A) with $^{32}P$ end-labeled HIV-κB oligonucleotide probe. Samples and lane numbers are as in (A). Lanes 11 and 12, competition controls performed on the same extract used in lane 3 (HEF cells, TNF-α, 15 min) with excess unlabeled wild-type and mutant oligonucleotide, respectively. (**C**) Gel shift of the same nuclear extracts as in (B) with the AP-1 consensus probe. Lane 11, competition with excess unlabeled oligonucleotide with the extract from lane 4. (**D**) Gel shift of the same nuclear extracts as in (B) with the Oct-1 probe. Lane 11, wild-type competition with the extract from lane 1.

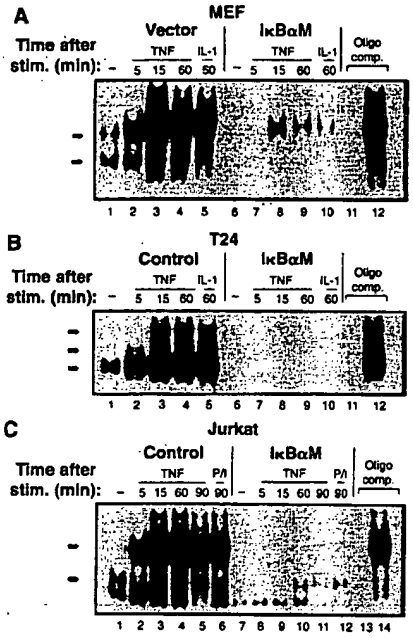


**Fig. 2.** Gel-shift analysis of various cell lines expressing IκBαM. Cell lines were stimulated with TNF-α, IL-1α, or PMA plus ionomycin. Nuclear extracts were prepared and analyzed for NF-κB activation by gel shift with $^{32}P$ end-labeled HIV-κB oligonucleotide. (**A**) Cells transduced with either empty LXSN vector or IκBαM were treated with TNF-α (10 ng/ml) or IL-1α (2 ng/ml). Lanes 1 to 5, control MEF cells; lanes 6 to 10, IκBαM MEF cells. (**B**) T24 cells were stimulated and treated as in (A). (**C**) Normal and IκBαM Jurkat cells treated with TNF-α (20 ng/ml) or PMA (40 ng/ml) plus ionomycin (1 μM). Lanes 1 to 6, control cells; lanes 7 to 12, IκBαM cells. Arrows indicate the shift (or shifts) corresponding to NF-κB. In each instance, mutant and wild-type unlabeled oligonucleotide competition was performed on the 15-min TNF-α–stimulated control sample.

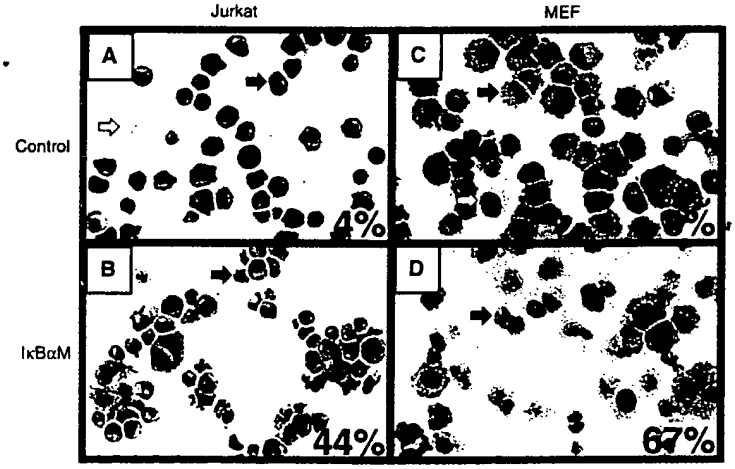


**Fig. 3.** Cytospin analysis of apoptosis. Cells were treated with TNF-α (100 ng/ml) for 18 hours, centrifuged onto slides, and stained with eosin–methylene blue to identify cells that had lost nuclear structure. Colored arrows indicate cells scored as apoptotic (red), necrotic (yellow), and viable (blue). (**A** and **C**) Control Jurkat and MEF cells, respectively. (**B** and **D**) Jurkat and MEF cells, respectively, transduced with IκBαM. The percentage of cells identified as apoptotic is indicated in the lower right of each panel. These values were obtained by averaging the results from two independent experiments in which five fields per slide with an average of ~100 cells per field were counted.

annexin V binding of control and IκBαM Jurkat cells obtained over time in the presence of a high dose of TNF-α (100 ng/ml) (*14*). Normal Jurkat cells showed only a slight increase in annexin V binding even at the longest incubation time, from 10.4% without stimulation to 30.1% after 48 hours. In contrast, the percentage of apoptotic IκBαM-expressing Jurkat cells increased from 12.3 to 39.9% in 7 hours, culminating in over 80% by 48 hours. The data on apoptosis were confirmed by use of light-scatter flow cytometric analysis (*15*). We further extended our results by showing that RelA (−/−) embryo fibroblasts (*16*) were more sensitive to TNF-α–induced apoptosis than either normal or p50 (−/−) fibroblasts (*17–19*).

By two independent criteria—the block of short- and long-term induction as shown by gel shift (Figs. 1B and 2) and the inhibition of endogenous IκBα resynthesis (Fig. 1A)—we have shown that IκBαM is a potent dominant-negative inhibitor of NF-κB activation. The finding that cells with a block in NF-κB signaling are more susceptible to TNF-α–induced apoptosis is consistent with observations that TNF-α cytotoxicity can be greatly enhanced by the addition of inhibitors of protein and RNA synthesis (for example, cyclohexamide and actinomycin D) (*2*). The same synergy of cell death signals has also been reported for Fas-induced cell death (*20*). Fas can induce NF-κB gel-shift activity in certain, but not all, cell types. T24, one of the cell lines shown to be capable of Fas-induced NF-κB activity, is also sensitive to Fas cytotoxity only in the presence of inhibitors of RNA or protein synthesis (*21*). We examined the effect of Fas activation on T24 cells expressing IκBαM and observed no appreciable cell death (*18*). Thus, it appears that the molecular mechanisms of Fas- and TNF-α–mediated cell death may be different, in that the activation of NF-κB can induce target gene expression that can rescue TNF-α–, but not Fas-mediated, apoptosis.

Inhibition of NF-κB may be used by organisms as a means of killing TNF-α–targeted cells. Inhibitors of NF-κB activation, such as glucocorticoids (*22*), antioxidants (*23*), and $Cu^{2+}$ (*24*), may fall in this category. Substantial therapeutic gains are possible if natural and synthetically derived inhibitors of NF-κB can be used in combination with TNF-α to treat conditions in which certain cells need to be cleared, such as cancer and bacterial and viral infection.

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6. Mouse embryo fibroblasts were derived from Swiss Webster mice as described (*26*) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human embryo fibroblasts were prepared and grown identically to MEF cells. Two hundred ninety-three cells expressing *gag* and *pol* were grown in DMEM, 10% FBS, and selection was maintained with blastacidin (20 μg/ml). T24 cells (American Type Culture Collection) and Jurkat cells were grown in RPMI 1640 in 10% FBS. All the pools of stable cells were produced in a similar manner. First, 293 cells stably expressing Moloney *gag* and *pol* under control of cytomegalovirus (CMV) promoter-enhancer were transiently transfected by $CaPO_4$ precipitation with 20 μg of pLXSN retroviral vector (*3*) and 5 μg of the plasmid pMDG (*27*) containing the vesicular stomatitis virus (VSVg) envelope downstream of the CMV promoter-enhancer. After 48 and 72 hours, the medium was removed, filtered, and either stored at −20°C or used immediately for infection. Infection was performed on ~$0.5 \times 10^5$ cells in 3 ml with Polybrene (8 μg/ml) for 8 to 12 hours. Cells were allowed to expand for 48 hours and were then selected for neomycin resistance. Amounts of G418 used in selecting the various cell types were as follows: MEF and HEF cells, 800 μg/ml; T24, 400 μg/ml; Jurkat, 1 mg/ml. Immunoblotting was then performed to analyze the expression of IκBαM.
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10. Electrophoretic mobility-shift assay was done as described (*29*). Briefly, 5 μg of nuclear extract were mixed with 0.5 μg of poly(dI-dC) and DNA binding buffer in a total volume of 10 μl and incubated on ice for 20 min. $^{32}P$-labeled oligonucleotides (6000 cpm per sample) were then added to the binding reaction and incubated for 30 min at room temperature. Competition was performed by addition of 10 ng of unlabeled oligonucleotide (500-fold excess) during the incubation on ice followed by addition of labeled oligonucleotide. The HIV-κB oligonucleotide has been described (*30*). Oct-1 and AP-1 consensus oligonucleotides were purchased from Santa Cruz Biotech. All the oligonucleotides were end-labeled by use of T4 polynucleotide kinase and [$\gamma$-$^{32}P$] adenosine triphosphate (Dupont NEN). TNF-α, IL-1α, phorbol 12-myristate 13-acetate (PMA), and ionomycin A23187 were obtained from Calbiochem.
11. Cytospin analysis was done as described (*31*). Briefly, cells were treated as described in the text, and a 100-μl sample was loaded into disposable chambers and centrifuged for 1 min onto glass slides in a cytocentrifuge (Shandon). The slides were allowed to dry and then stained with the Leukostat stain kit (Fisher Scientific). Staining was verified by visualization under a light microscope, and the slides were mounted with glass cover slips with the use of Permount mounting media (Fisher Scientific). The population of cells was categorized as either viable, apoptotic, or necrotic when analyzed by staining morphology. Counting was performed blind, meaning that each slide was given a number and the identity of each sample was not known during counting. The counting procedure made use of a light microscope at a magnification that allowed the incorporation of about 100 cells per field. Five fields were counted per slide, and each experimental condition was performed two times.
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